L-Ascorbic Acid Increases NFκB Binding Activity in UVA-Irradiated HaCaT Keratinocytes

To the Editor:

The biologic effects of UV on skin may occur either as an acute alteration shortly after UV exposure (inflammation, sunburn cells) or as a chronic damage (photoaging, photocarcinogenesis) (Beissert and Granstein, 1996). Reactive oxygen species (ROS) are involved in the pathophysiologic mechanisms leading to photooxidative damage on nucleic acids, lipids, and proteins. ROS are also responsible for the induction of proinflammatory cytokines and adhesion molecules (Punnonen *et al*, 1991; Krutmann and Grewe, 1995). ROS are constantly generated in epidermal keratinocytes and are rapidly removed by nonenzymic and enzymic antioxidant substances that prevent harmful effects of free radicals and maintain a prooxidant/antioxidant balance, thus resulting in cell and tissue stabilization (Fuchs *et al*, 1989; Yohn *et al*, 1991).

The intracellular pathway of ROS signal transduction is not fully understood. The nuclear transcription factor kappa B (NFκB) may be one candidate for the intracellular response of UVA-induced ROS in keratinocytes. NFκB is an inducible transcription factor involved in the regulation of genes participating in either inflammatory or immune responses. In most cell types NFκB resides in the cytoplasm in a latent form composed of several subunits. NFκB activation occurs by dissociation from its inhibitor protein (IκB) and its translocation into the nucleus (Baldwin, 1996). NFκB can be activated by various agents including UV. The fact that NFκB activation may be inhibited by antioxidants, suggests that ROS are involved in activating NFκB (Devary *et al*, 1993; Bender *et al*, 1998).

Activation of NFκB can be induced in human skin by UVB (Fisher *et al*, 1996). In cultured human keratinocytes NFκB activation occurs after combined UVA/UVB irradiation and also in cultured fibroblasts UVA irradiation activates NFκB; however, high UVA doses lead to decreased NFκB (Vile *et al*, 1995; Djavaheri-Mergny *et al*, 1999; Saliou *et al*, 1999). UVA-induced upregulation of interleukin-1α (IL-1α) can be inhibited by L-ascorbic acid (Tebbe *et al*, 1997). In this investigation, we tested the influence of L-ascorbic acid on NFκB binding activity, in order to obtain a linkage between UVA-induced ROS and immune response in keratinocytes.

HaCaT cells, an immortalized aneuploid human keratinocyte cell line (Boukamp *et al*, 1988) shows a higher constitutive NFκB binding activity than normal keratinocytes. The response to some cell cycle regulating factors in normal keratinocytes and HaCaT cells has been compared (Chaturvedi *et al*, 1999; Qin *et al*, 1999). A strong activation of NFκB by factors inducing cell cycle arrest is only observed in normal keratinocytes. The absence on responsiveness to cell cycle regulatory factors correlates well with the immortalized state of HaCaT cells. In spite of the constitutive level of binding activity present in HaCaT, these cells provide a homogenous system that allow to compare other NFκB inducing agents. Saliou *et al* (1999) showed in HaCaT cells that NFκB activation after UV radiation can be modulated by different agents. These studies are not possible to do by using a highly heterogeneous system like human normal keratinocytes coming from different individuals.

UVA irradiation increases TBARS formation in HaCaT keratinocytes. This effect can be partially inhibited by L-ascorbic acid indicating that ROS are involved (data not shown). NFκB binding acitivity, measured in the nuclear protein extract of HaCaT keratinocytes, is only increased within a short time period after UVA irradiation (**Fig 1**). L-ascorbic acid supplementation and UVA irradiation (1 J per $cm^{2)}$ showed a synergistic effect on NFκB binding activity in HaCaT keratinocytes. NFκB binding activity was found to be increased to 156% (SEM ± 19%) in UVA-irradiated cells. L-ascorbic acid alone did not change NFκB binding activity; however, UVA irradiation-increased NFκB binding activity in L-ascorbic acid supplemented cells showed remarkable increase (**Fig 2*a*, *b***). Both NFκB subunits p50 and p65 were detectable in nuclear protein extracts of HaCaT keratinocytes (**Fig 2*c***).

The regulation of NFκB by ROS is cell type specific. In certain cell types, such as Wurzburg subclone of T cells, L6 skeletal muscle myotubes, human breast CF-7, and 70Z/3 pre-B cells, H_2O_2 was shown to be an effective inducer of NFκB-binding activity (Sen and Packer, 1996; Manna *et al*, 1998). It has been reported that H_2O_2 can also induce NFκB binding activity in HeLa cells and Jurkat cells (Schreck *et al*, 1991; Brennan and O'Neill, 1995);

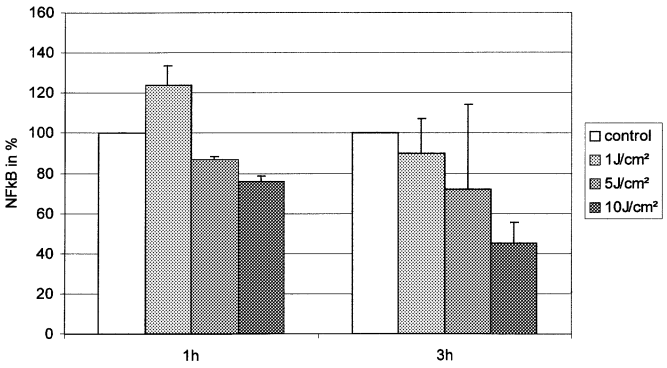

Figure 1. Highest NFκB binding activity in HaCaT cells was seen 1 h after UVA irradiation (1 J per cm^2). HaCaT cells were irradiated with 1, 5, or 10 J per cm^2. UVA irradiation was performed from the bottom of the cell culture dishes using a lamp with a range of 320–400 nm (Waldmann, Villingen, Germany). The output measured by a radiometer through culture dish was 6.7 mW per cm^2. Nuclear proteins were extracted from the cultured cells according to Corsini *et al* (1997). Electrophoretic mobility shift assays (EMSA) were performed according to Hirano *et al* (1998). A double-stranded oligonucleotide containing the NFκB binding site (sense 5′-AGCTTCAGAGGGGACTTTCCGAG-AGG-3′, antisense 5′-TCGACCTCTCGGAAAGTCCCCTCTGA-3′) was labeled with [α-^{32}P]dCTP (NEN Life Science Products, Boston, MA) using Klenow Fragment (Gibco BRL, Gaithersburg, MD). Data for NFκB binding activity were quantified using computer-based densitometry (TINA).

Manuscript received August 22, 2000; revised February 8, 2001; accepted for publication February 23, 2001.

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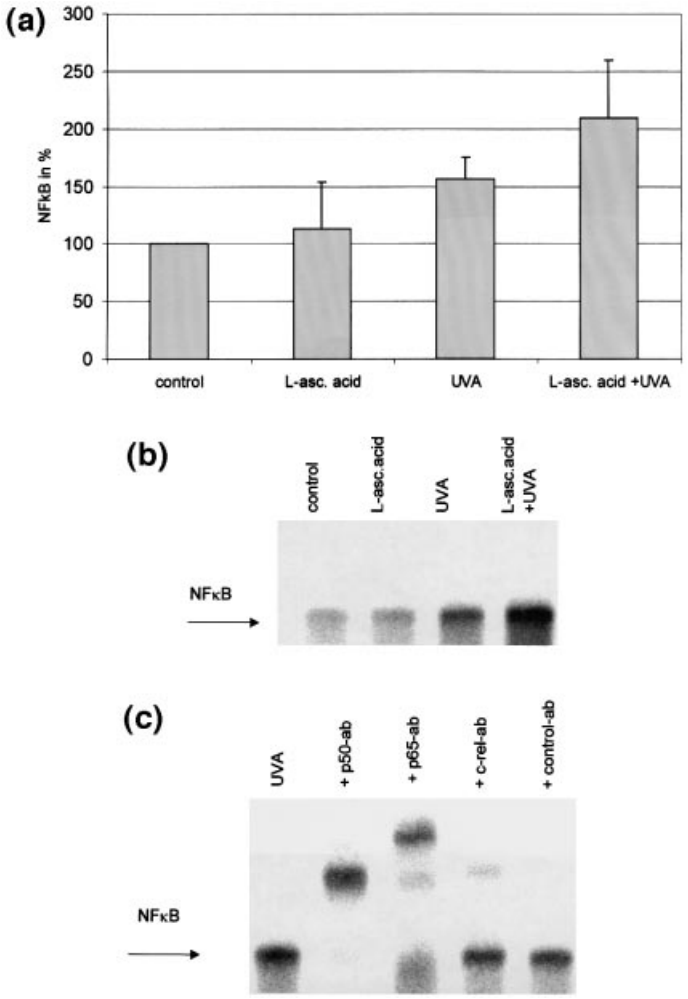

Figure 2. (*a*, *b*) L-ascorbic acid increases UVA-induced NFκB binding activity in HaCaT keratinocytes. NFκB binding activity was measured by EMSA in UVA-irradiated (1 J per cm^2) HaCaT keratinocytes supplemented with L-ascorbic acid (10^{-4}M). NFκB binding activity was quantified using computer-based densitometry and related to nonirradiated HaCaT keratinocytes without L-ascorbic acid supplementation. UVA-induced NFκB binding acitivity was markedly increased by L-ascorbic acid (mean ± SEM). **(*c*) NFκB subunits p50 and p65 are both expressed in HaCaT cells after UVA irradiation.** NFκB supershift assay for detection of p50, p65, and c-rel subunits was used in HaCaT keratinocytes irradiated with UVA (1 J per cm^2). Both p50 and p65 specific antibodies were found positive, whereas c-rel was not detectable. Specific antibodies directed against NFκB subunits p50, p65, c-rel (Santa Cruz Biotechnology, Santa Cruz) were used for supershift assay.

however, some other groups could not detect NFκB binding activity in these cell lines after H_2O_2 stimulation (Anderson *et al*, 1994; Li and Karin, 1999).

The effect of ascorbic acid on NFκB activation varies in different cell types. Ascorbate increases the binding of NFκB to DNA in TNF-α stimulated Jurkat cells. The ability of ascorbate to enhance cytoplasmic inhibitory IκBα protein degradation correlates completely with its capacity to induce NFκB binding to DNA that potentiate NFκB-mediated transactivation of the HIV-1 long-terminal repeat (Muñoz *et al*, 1997).

Beside its antioxidative properties, ascorbic acid can also interact with metal ions that contribute to oxidative damage through the production of hydroxyl and lipid alkoxyl radicals (Carr and Frei, 1999). Ascorbate increases NO production by increasing the amount of iNOS in activated macrophages (Mizutani and Tsukagoshi, 1999). This is caused by a delay of IκB recovery and prolonged NFκB DNA binding in lipopolysaccharide and interferon-γ activated macrophages leading to an increase in the iNOS protein level (Mizutani and Tsukagoshi, 1999).

HaCaT keratinocytes possess very efficient systems to maintain high levels of both intracellular and extracellular ascorbic acid (Savini *et al*, 1999). The regeneration and uptake of ascorbic acid from extracellular medium contributes to the intracellular antioxidant capacity, and consequently cells become more resistant to free radical generation and cell death induced by UVB irradiation (Savini *et al*, 1999).

Our results indicate that UVA induces NFκB binding activity in HaCaT keratinocytes, and this effect can be increased by L-ascorbic acid indicating that the latter has either an effect on IκB degradation or prooxidative properties. Redox regulation of cell nutrients may induce changes in cell signalling or protein conformation that represent an important mechanism in the regulation of transcription factors and gene expression.

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REFERENCES

Anderson MT, Staal FJ, Gitler C, Herzenberg LA, Herzenberg A: Separation of oxidant-initiated and redox-regulated steps in the NF-kappa B signal transduction pathway. *Proc Natl Acad Sci (USA)* 91:11527–11531, 1994

Baldwin AS: The NF-kappa-B and I-kappa-B proteins – New discoveries and insights. *Annu Rev Immunol* 14:649–683, 1996

Beissert S, Granstein RD: UV-induced cutaneous photobiology. *Crit Rev Biochem Mol Biol* 31:381–404, 1996

Bender K, Göttlicher M, Whiteside S, Rahmsdorf HJ, Herrlich P, Sequential DNA: damage-independent and –dependent activation of NF-κB by UV. *EMBO J* 17:5170–5181, 1998

Boukamp P, Petrussevska RT, Breitkreutz D, Hornung J, Markham A, Fusenig NE: Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line. *J Cell Biol* 106:761–771, 1988

Brennan P, O'Neill LAJ: Effects of oxidants and antioxidants on nuclear factor κB activation in three different cell lines: evidence against a universal hypothesis involving oxygen radicals. *Biochimica Biophysica Acta* 1260:167–175, 1995

Carr A, Frei B: Does vitamin C act as pro-oxidant under physiological conditions? *FASEB J* 13:1007–1024, 1999

Chaturvedi V, Qin JZ, Denning MF, Choubey D, Diaz MO, Nickoloff BJ: Apoptosis in proliferating, senescent, and immortalized keratinocytes. *J Biol Chem* 274:23358–23367, 1999

Corsini E, Terzoli A, Bruccoleri A, Marinovich M, Galli CL: Induction of tumor necrosis factor-α in vivo by a skin irritant, tributytin, through activation of transcription factors: its pharmacological modulation by antiinflammatory drugs. *J Invest Dermatol* 108:892–896, 1997

Devary Y, Rosette C, DiDonato JA, Karin M: NF-kappa-B activation by ultraviolet light not dependent on a nuclear signal. *Science* 261:1441–1445, 1993

Djavaheri-Mergny M, Gras MP, Mergny JL, Dubertret L: UV-A-induced decrease in nuclear factor-κB activity in human keratinocytes. *Biochem J* 338:607–613, 1999

Fisher GJ, Datta SC, Talwar HS, Wang ZQ, Varani J, Kang S, Voorhees JJ: Molecular basis of sun-induced premature skin ageing and retinoid antagonism. *Nature* 379:335–339, 1996

Fuchs J, Huflejt ME, Rothfuss LM, Wilson DS, Carcamo G, Packer L: Impairment of enzymic and nonenzymic antioxidants in skin by UVB irradiation. *J Invest Dermatol* 93:769–773, 1989

Hirano F, Chung M, Tanaka H, Maruyama N, Makino I, Moore DD, Scheidereit C: Alternative splicing variants of IκBβ establish differential NF-κB signal responsiveness in human cells. *Mol Cell Biol* 18:2596–2607, 1998

Krutmann J, Grewe M: Involvement of cytokines, DNA damage, and reactive oxygen intermediates in ultraviolet radiation-induced modulation of intercellular adhesion molecule-1 expression. *J Invest Dermatol* 105:67S–70S, 1995

Li N, Karin M: Is NF-κB the sensor of oxidative stress? *FASEB J* 13:1137–1143, 1999

Manna S, Zhang HJ, Yan T, Oberley LW, Aggarwal BB: Overexpression of manganese superoxide dismutase suppresses tumor necrosis factor-induced apoptosis and activation of nuclear transcription factor-kappaB and activated protein-1. *J Biol Chem* 273:13245–13254, 1998

Mizutani A, Tsukagoshi N: Molecular role of ascorbate in enhancement of NO production in activated macrophage-like cell line, J774.1. *J Nutr Sci Vitaminol (Tokyo)* 45:423–435, 1999

Muñoz E, Blázquez MV, Ortiz C, Gomez-Díaz C, Navas P: Role of ascorbate in the

activation of NF-κB by tumor necrosis factor α in T-cells. *Biochem J* 325:23–28, 1997

Punnonen K, Puntala A, Ahotupa M: Effects of ultraviolet A and B irradiation on lipid peroxidation and activity of the antioxidant enzymes in keratinocytes culture. *Photodermatol Photoimmunol Photomed* 8:3–6, 1991

Qin JZ, Chaturvedi V, Denning MF, Choubey D, Diaz MO, Nickoloff B: Role of NF-κB in the apoptotic-resistent phenotype of keratinocytes. *J Biol Chem* 274:37957–37964, 1999

Saliou C, Kitazawa M, McLaughlin L, *et al:* Antioxidants modulate acute solar ultraviolet radiation induced NF-Kappa-B activation in a human keratinocyte cell line. *Free Rad Biol Med* 26:174–183, 1999

Savini I, D'Angelo, I, Ranalli M, Melino G, Avigliano L: Ascorbic acid maintenance in HaCaT cells prevents radical formation and apoptosis by UV-B. *Free Radic Biol Med* 26:1172–1180, 1999

Schreck R, Rieber P, Baeuerle PA: Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF-kappa B transcription factor and HIV-1. *EMBO J* 10:2247–2258, 1991

Sen CK, Packer L: Antioxidant and redox regulation of gene transcription. *FASEB J* 10:709–720, 1996

Tebbe B, Wu S, Geilen CC, Eberle J, Kodelja V, Orfanos CE: L-Ascorbic acid inhibits UVA-induced lipid peroxidation and secretion of IL-1α and IL-6 in cultured human keratinocytes in vitro. *J Invest Dermatol* 108:302–306, 1997

Vile GF, Tanew-Iliitschew A, Tyrrell RM: Activation of $NF_{\kappa}B$ in human skin fibroblasts by the oxidative stress generated by UVA radiation. *Photochem Photobiol* 62:463–468, 1995

Yohn JJ, Norris DA, Yrastorza DG, Buno IJ, Leff JA, Hake SS, Repine JE: Disparate antioxidant enzyme acitvities in cultured human cutaneous fibroblasts, keratinocytes, and melanocytes. *J Invest Dermatol* 97:405–409, 1991

The Pro162 Variant is a Loss-of-Function Mutation of the Human Melanocortin 1 Receptor Gene

To the Editor:

α melanocyte-stimulating hormone (αMSH) stimulates mammalian melanocytes differentiation and/or proliferation (Abdel-Malek *et al*, 1995), by binding to the G protein-coupled melanocortin 1 receptor (MC1R) and triggering the cAMP cascade. The gene encoding for human MC1R was simultaneously cloned by Mountjoy *et al* (1992) and Chhajlani and Wikberg (1992), who reported slightly different sequences. Mammalian pigmentation depends, among other factors, on the relative amounts of skin eumelanin and pheomelanin (Thody and Graham, 1998). In man, MC1R loss-of-function mutations, such as Arg151Cys, are causally associated with predominantly pheomelanic red hair (reviewed by Rees, 2000). Because red hair is a risk factor for increased UV radiation skin sensitivity, a relationship of *MC1R* allelic variants and skin cancer susceptibility has been investigated. Evidence has been presented for an association between the Arg151Cys, Arg160Trp, and Asp294His variants, red hair, and melanoma (Palmer *et al*, 2000).

The study of MC1R structure–function relationships and its association with complex traits is complicated by the high polymorphism of the gene (Rees, 2000). The functional effects of many amino acid substitutions are debated or unknown. Moreover, the reported wild-type sequences differ in potentially relevant residues. An intriguing variant, originally reported as a wild-type allele, displays a proline at position 162 (Mountjoy *et al*, 1992), in the interface between the second cytosolic loop and the fourth transmembrane fragment, instead of an arginine residue reported by others (Chhajlani and Wikberg, 1992; Tan *et al*, 1999). Because Pro residues distort helical structures, an Arg/Pro substitution at the beginning of a transmembrane fragment could be functionally important.

In a survey of *MC1R* variants in cultured human melanoma and giant congenital nevus cells, performed by sequencing the entire coding sequence, we failed to find the Pro162 allele in 10 samples (**Fig 1*A***, consensus sequence deposited in GenBank, number AF326275). Unfortunately, no data are available as to the pigmentation phenotypes of the patients/donors. This prompted us to screen a larger number of samples by allele-specific PCR (AS-PCR). Thirty healthy individuals were selected, of which 13 had fair skin and poor tanning ability, whereas 17 had black hair and a high basal pigmentation and tanning response. We used an artificial

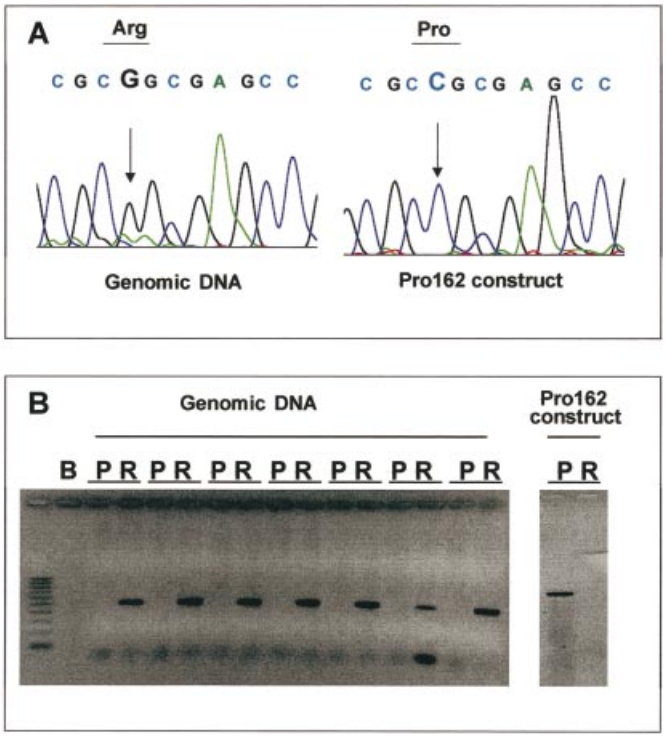

Figure 1. Identification of the Arg162 allele as the wild-type form of *MC1R*. The *MC1R* coding sequence was amplified from genomic DNA using *Pfu* polymerase and primers CCTAAGCTTACTCC-TTCCTGCTTCCTGGACA (forward, nt 435–456) and CTG-GAATTCACACTTAAAGCGCGTGCACCGC (reverse, nt 1418–1439), designed from the sequence reported by Mountjoy *et al* (1992), GenBank entry X65634, and containing added *Hind* III and *EcoR* I sites (underlined) for cloning in pcDNA3 (Invitrogen, Carlsbad, CA). (*A*) Sequence of amplicons from a human melanoma cell line (left), and a Pro162 construct (right), used as sequencing quality control. The relevant codon is highlighted, and the mutated base shown by an *arrow*. (*B*) Inability to detect the Pro162 variant by AS-PCR of genomic DNA. Forward primers TGACCCTGCCGCGGGCGC**G** (R, specific for the Arg162 form) and TGACCCTGCCGCGGGCGC**C** (P, for the Pro162 variant), where the nucleotide responsible for specificity is shown in bold, were used with the reverse primer described above, and 1 μg of genomic DNA or 12 pg of the Pro162 pcDNA3 construct as target. PCR reactions with the specific primer sets for Pro (P) and Arg (R) alleles, as indicated over each lane, were analyzed by agarose gel electrophoresis. A blank reaction, without added target (labeled B), and a 100 bp ladder (first lane, left) are shown.

Manuscript received January 25, 2001; revised March 8, 2001; accepted for publication March 15, 2001.

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